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Award Number: W81XWH-11-2-0111

TITLE: Early Life Stress and Sleep Restriction as Risk Factors in PTSD: An Integrative Pre-Clinical Approach

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REPORT DATE: April 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-04-2012	Annual	1 APR 2011 - 31 MAR 2012

4. TITLE AND SUBTITLE
Early Life Stress and Sleep Restriction as Risk Factors in PTSD: An Integrative Pre-Clinical Approach

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-2-0111

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Haifa
Haifa 31905

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Abstract on next page.

15. SUBJECT TERMS
Subject terms on next page.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	45	19b. TELEPHONE NUMBER *(include area code)*

14. ABSTRACT
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1) To establish an effective animal model of PTSD that would consider the contribution of risk factors to the induction of the trauma.
2) To examine the role of sleep restriction as an immediate risk factor in PTSD.
3) To establish the role of childhood adversity as a long-term risk factor in PTSD, particularly in association with sleep restriction.
4) To develop the model as a platform for pharmacological testing of novel targets for drug development.

15. SUBJECT TERMS
Post-traumatic Stress Disorder; Risk factors; Animal model; Drug testing platform.

Title: **Early life stress and sleep restriction as risk factors in PTSD – An integrative preclinical approach**

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Introduction

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Why risk factors? - Because PTSD is the only psychiatric disorder to which there is seemingly a clear etiological agent – a traumatic event that triggers it, most models of PTSD concentrate on what would constitute a trauma in the studied animals. However, because the majority of people exposed to traumatic experiences actually do not develop PTSD the exposure to the traumatic experience is necessary, but not a sufficient condition to induce the disorder. We focus on both distal (Childhood adversities) and proximal (Sleep restriction) potential risk factors, with high relevance to soldiers. The primary aims of the project are thus:

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Body

Task 1: **Generation of an approved animal use protocol –**

Animal Use Protocol was approved both by the Institute's IACUC as well as by the US Army Animal Care and Use Review Office. All experiments were conducted only after approval of the protocol.

Task 2. **The establishment of sleep restriction methodology in Haifa –**

We have adopted the well established protocol of Meerlo et al, (2008). In order to validate that this model works well in our hands, we have conducted a telemetry study, using a combination of a DSI telemetry system and the sleep restriction wheels system we have set for this project.

Aim: The aim of the experiment was to establish a protocol for future studying the physiological and behavioral effects of sleep restriction on coping with Under Water Trauma (UWT) in rats.

Methods:

Animals –

Male Sprague Dawley rats (~36 days old, 125-150 g) were used for the experiment. Animals were housed at 22 ± 2°C under 12-h light/dark cycles. Water and food were available ad libitum. After acclimation they were individually housed.

Experimental groups

Following acclimation all rats were randomly assigned to one of the following experimental conditions:

1. Sleep restriction (SR) – Rats exposed to 8 days of SR.
2. Sleep restriction Control (control) – Rats exposed to the control procedure of SR.

Experimental design

Following delivery and acclimation to the vivarium, rats of all tested groups were implanted at ~45 PND with radio transmitters (model TA10TA-F40; Data Sciences Inc., St Paul, MN, USA), that enable monitoring of core body temperature and activity level. After surgery, animals had at least 7 days to recover, before the telemetry recordings had began. Rats were than habituated to the SR apparatus by placing them on the wheels (will be described in the section of SR procedure) for 1 h on 3 consecutive days (Slowly or voluntary rotating wheels, according to the experimental group). At ~60 PND, some of the rats were exposed to SR for 8 days and the others were exposed to the control procedure of SR. After 8 days of SR the rats were assessed cognitively using the Object location recognition task and than their ability to learn under stress was assessed using the Two way shuttle avoidance (TWSA) task. 2-3 consecutive days before the Object location recognition task the rats were habituated to the Open field arena during the first hours on the wheels. The TWSA task was given twice in 2 consecutive days, while in between they had one more day of SR or its control procedure (all in all, SR actually lasted 9 days). Telemetry recordings were taken for 10 more days. Timeline of procedures is presented in figure 1.

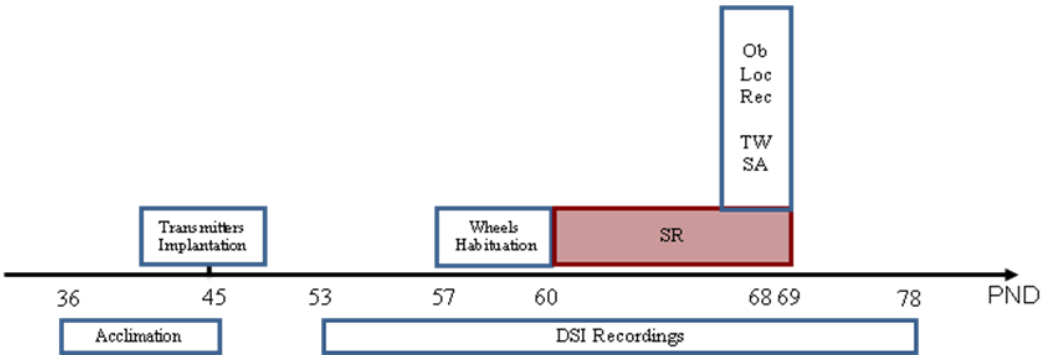

Figure 1: Timeline of procedures.

Procedures and assessments

Circadian monitoring of activity and body temperature

Rats of all tested groups were implanted at the age of approximately 45 days with radio transmitters (model TA10TA-F40; Data Sciences Inc., St Paul, MN, USA), that enable monitoring of body

temperature and activity level. Anesthesia for the implantation was induced by a mixture of 100mg/Kg Ketamine together with 2% Xylazin. Rats were also injected with 5% Rimadyl to reduce pain and with 15% Amoxycillin to prevent possible infections (Rimadyl and Amoxycillin were also injected for two additional days after surgery). Transmitters were placed in the abdominal cavity. After surgery, animals had 7-8 days to recover. Body temperature and activity levels were than recorded for 28 days, using receivers (model RPC-1; DSI) placed under home cages that relay the radio signals to a PC. Recordings included 5 days of baseline, 3 days of SR habituation, 9 days of SR procedure, and 10 more days of follow-up. Data was sampled for 5-30s once every 5 min. Activity level while animals were on the wheels was not taken into consideration since while running on the wheel DSI calculates almost no activity in reference to the position of the animal on the receiver. For technical reasons recordings were taken only when the animals were not on the activity wheels on the first batch, but on the next two batches this was solved and recordings were taken all through the SR procedure.

Sleep restriction

We adopted the well established SR protocol of Meerlo et al., (2002) in rats. SR was performed by confining the rats in slowly rotating wheels (diameter 35.5 cm, approximately one meter per minute, model 80860A; Lafayette Instruments Company, Lafayette, IN, USA). Control rats were placed in voluntary rotating wheels (model 80860W). The rats had continuous access to food and water at the side of the wheel. They were allowed to sleep in their home cage between 9:00-13:00, the first 4 hrs of the light phase (i.e. the first 4 hrs of their normal resting phase). The remaining 20 hrs of the day, the sleep restricted rats were placed in the motorized rotating wheels and control rats were placed in the voluntary rotating wheels. Since rats normally sleep approximately 12 hrs each day (Borbely & Neuhaus, 1979), 4 hrs of sleep are not sufficient to fully recover from the 20 hrs of wakefulness (Meerlo et al., 2002). This protocol does not induce total sleep deprivation but a significant sleep restriction and reduced sleep quality.

Object location recognition task

This task was adopted from Barker & Warburton (2011). We assessed the rats' ability to recognize that an object they had experienced before had changed location.

- *Habituation.* Rats were first habituated to the open field arena for 10 min on 2-3 consecutive days, during the last sessions of SR. The open field consists of a black box 90.0cm × 90.0cm × 38.0cm, positioned in a dimly-lit room. Each rat was placed in the center of the open field for 10 min of free exploration. Total distance moved, distance moved, time spent, and the number of enters to the central area was recorded using a video tracking software (EthoVision XT 8; Noldus Information Technology). The total distance moved represents the activity level of the rat. The

relative distance moved, and time spent in the center and the number of enters, are considered a measure of anxiety.

- *Acquisition phase.* In the acquisition phase two identical objects were placed in the far corners of the open field arena. After 5 min of habituation to the room the rat was placed in the center of the arena facing the opposite wall from the objects and allowed to explore both objects during a sample phase of 5 min. The amount of exploration of each object was recorded. Rats that explored the objects less than 15s were excluded. The acquisition phase was carried out between 9:00-12:00 and the rats were kept on their wheels right until it began.
- *Test phase.* In the test phase, after a delay of 40 min, one object was placed in the same position as in the sample phase and the other object was placed in the corner adjacent to the original position, so that the two objects were diagonal from each other. Thus, both objects in the test phase were equally familiar, but one was in a new location. The position of the moved object was counterbalanced between rats.

Two-way shuttle avoidance learning task

- *Apparatus.* (adapted from Tsoory and Richter-Levin, 2006): The TWSA box, placed in a dimly-lit, ventilated, sound-attenuated cupboard, is a rectangular chamber (60X26X28 cm) divided by an opaque partition with a small passage (10X8 cm) connecting two equal sized side-by-side cube-shaped compartments. The metal grid floors of both compartments are weight sensitive; micro-switches transmit information about the rat's location to a computer-controlled and automated data collection program which controls both CS (Conditioned Stimulus) presentations - a tone produced by loudspeakers located on the distal walls of the compartments, and US (Unconditioned Stimulus) presentations - electric shock, as well as recording the rats responses.
- *Procedure*. The task was carried out between 10:00-14:00, a few minutes to 45 minutes after the Object location recognition test phase on the first day. Rats went through 75 conditioning trials in each day. CS: maximum of tone; 10 sec; US: immediately following the CS termination an electric shock (0.8 mA) delivered for a maximum of 10 sec; ITI: randomly varying 30 sec ± 20%. The first day session started with 10 minutes of free exploration of the apparatus.

Rats can produce one of three responses:

Avoidance – shuttling to the adjacent compartment upon hearing the CS; following shuttling to the adjacent compartment the tone is stopped and an ITI commences; the rat avoids the electric shock.

Escape – shuttling to the adjacent compartment while the shock is on; the shock is stopped and an ITI commences; the rat only reduces the duration it is exposed to the shock.

Escape failure – failing to move to the adjacent compartment; the ITI commences at the completion of the 10 sec. foot shock, so the rat is subjected to the full duration of the electric shock.

Statistical Analysis

Differences were determined using Repeated measures analysis of variance (ANOVA), T-test, Sign test or Mann-Whitney U test.

Results

Activity levels while on the activity wheels during the SR protocol

As depicted in figure 2, Repeated measures ANOVA indicated a significant main effect for group on the total rotations per hour on the activity wheels during 8 days of SR [$F_{(1,19)}$= 10.05, $p<0.01$]. This effect was significant only during the light phase but not during the dark phase [$F_{(1,19)}$= 99.06, $p<0.001$; $F_{(1,19)}$= 0, NS, respectively]. During the light phase SR group was forced to be more active than the control group, but during the dark phase there was no difference in the general level of activity. This result indicates A) that the control group had a normal circadian activity cycle, and B) that, as required, this protocol disrupted the rest period of the SR group compared to the control group.

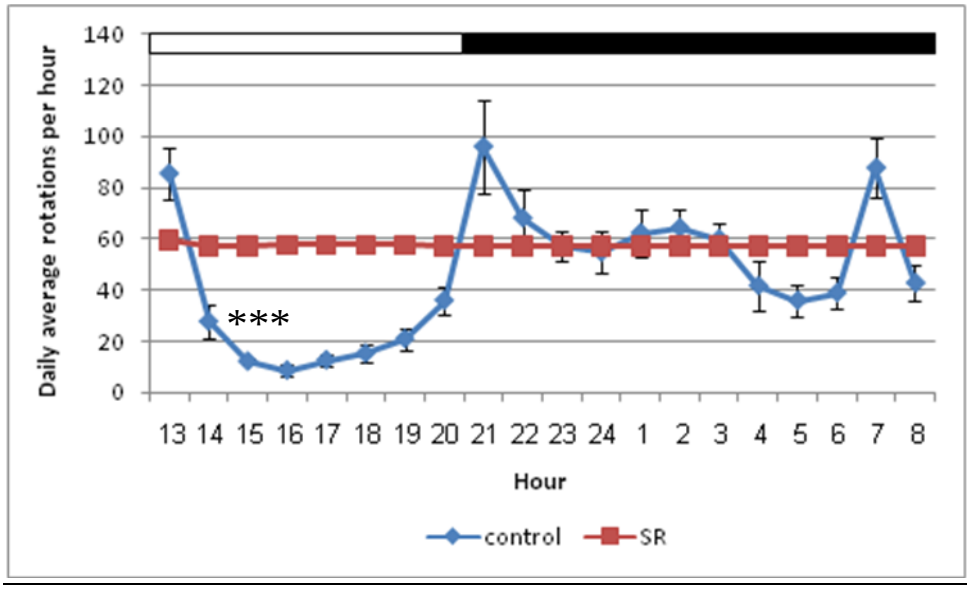

Figure 2: Rotations per hour in the activity wheels during 8 days of SR**:** During the light phase the SR group was forced to be more active than the control group but there was no difference between groups during the dark phase. The horizontal white and black bars represent light and dark, respectively.

[N: control – n=10, SR – n=11. (*** significant difference between groups, p<.001)].

Circadian activity level and body temperature

Circadian activity level: Repeated measures ANOVA indicated no significant differences in the averaged circadian activity level between groups as measured by the DSI during baseline or the SR days (while not on the wheels) [$F_{(1,24)}$= 1.36, NS; $F_{(1,23)}$= 1.68, NS, respectively]. But as depicted in figure 3, during the follow-up recording period, there was a significant difference between groups for the averaged circadian activity level as measured by the DSI [$F_{(1,24)}$= 6.1, $p<0.05$]. Further analysis indicated that the difference between groups was significant only during the dark phase but not during the light phase [$F_{(1,24)}$= 6.81, $p<0.05$; $F_{(1,24)}$= 0.93, NS, respectively]. Apparently following the SR procedure, SR group was less active than the control group during the dark phase which is normally the rats' active phase.

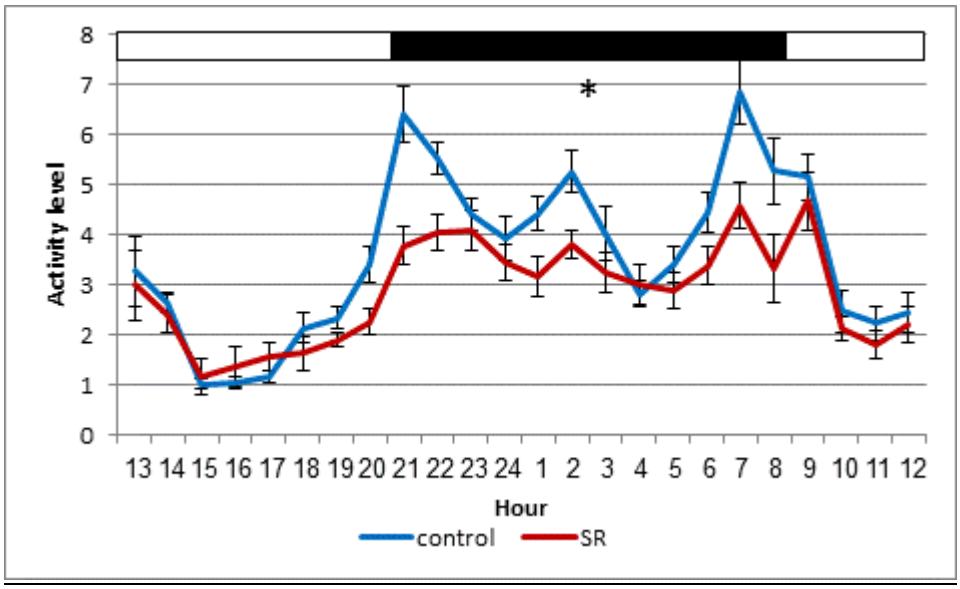

Figure 3: Averaged circadian activity level as measured by the DSI during the follow-up days: During the follow-up days there was a significant difference between groups. During the dark phase SR group was significantly less active than the control group, while no difference was found during the light phase. The horizontal white and black bars represent light and dark, respectively.

[N: control – n=14, SR – n=12. (* significant difference between groups, p<.05)].

Circadian body temperature: Repeated measures ANOVA indicated significant differences for the averaged circadian body temperature between groups only during SR days and not during baseline or follow-up [F(1,12)= 25.85, $p<0.001$; F(1,24)= 0.53, NS; F(1,24)= 0.60, NS, respectively]. As depicted in figure 4, during SR days, body temperature of rats in the SR group was significantly higher than that of the control group.

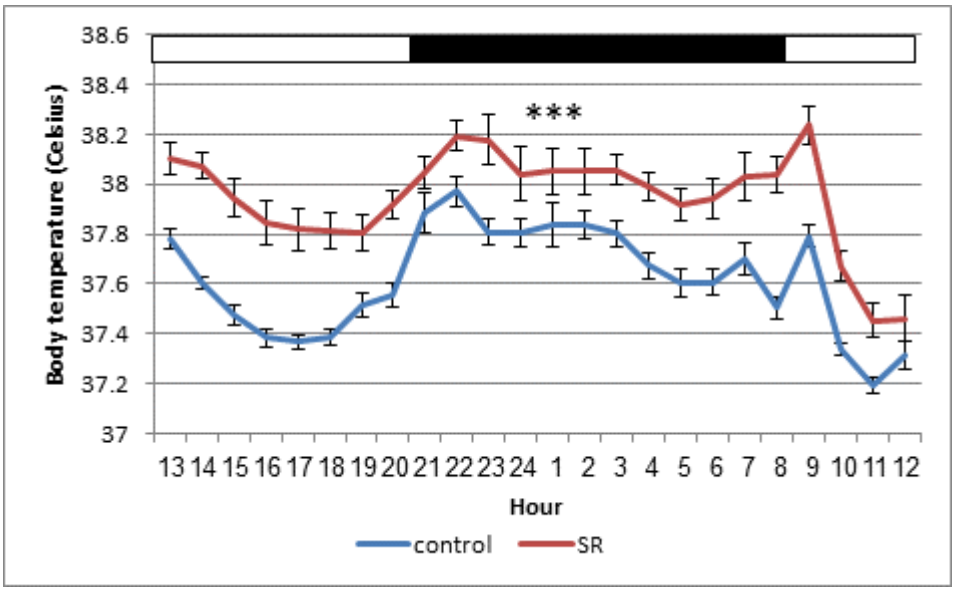

Figure 4: Averaged body temperature during the SR days: Body temperature of rats in the SR group was higher than that of the control group. The horizontal white and black bars represent light and dark, respectively.
[N: control - 8, SR - 6. (*** significant difference between groups, p<.001)].

Within the control group, Repeated measures ANOVA indicated significant difference in the average circadian body temperature between SR days and baseline, but not between follow up and baseline [$F(1,7)= 74.29$, $p<0.001$; $F(1,13)= 3.77$, NS], respectively, as depicted in figure 5. Further analysis showed that comparing to baseline, during the SR days, control animals had higher body temperature during the light phase, only when they were on the wheels and not while in their home cages [$F(1,7)= 112.57$, $p<0.001$; $F(1,13)= 0.79$, NS], respectively; and lower body temperature during the dark phase [$F(1,7)= 18.05$, $p<0.01$]. Apparently during SR days the control animals' body temperature was affected while they were on the activity wheels, but the SR protocol did not affect their body temperature at all at the following days.

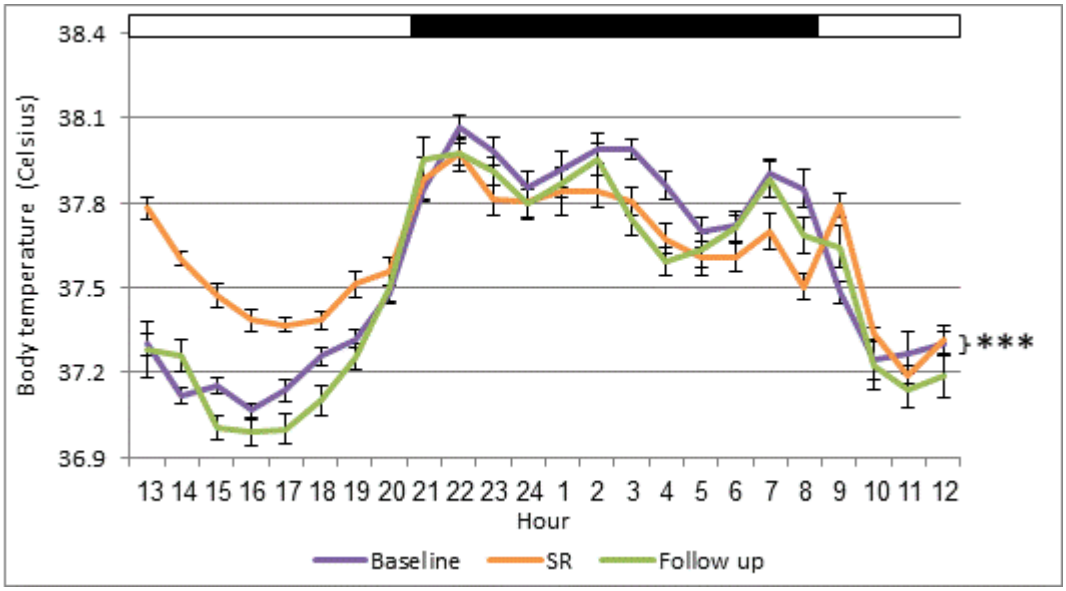

Figure 5: Average circadian body temperature within the control group: control animals' body temperature was higher than baseline during the light phase of SR days, only when they were on the wheels and lower than baseline during the dark phase of SR days. Control animals' body temperature during follow up was not different than baseline. The horizontal white and black bars represent light and dark, respectively.
[N: control - 14 (*** significant difference between baseline and SR days, p<.001)].

Within the SR group, Repeated measures ANOVA indicated significant difference in the average circadian body temperature between SR days and baseline, as well as between follow up and baseline [$F(1,6)= 78.53$, $p<0.001$; $F(1,11)= 18.82$, $p=0.001$], respectively, as depicted in figure 6. Further analysis showed that SR animals had higher body temperature during the SR days comparing to baseline. In addition these animals had lower body temperature during the follow up comparing to baseline, only during the dark phase and not during the light phase [$F(1,11)= 5.81$, $p<0.05$; $F(1,11)= 0.59$, NS], respectively. Apparently SR protocol has raised the SR animals body temperature during the procedure, and lowered body temperature on the follow-up days during the dark phase, while the animals were less active (as shown by the activity level data indicated above.)

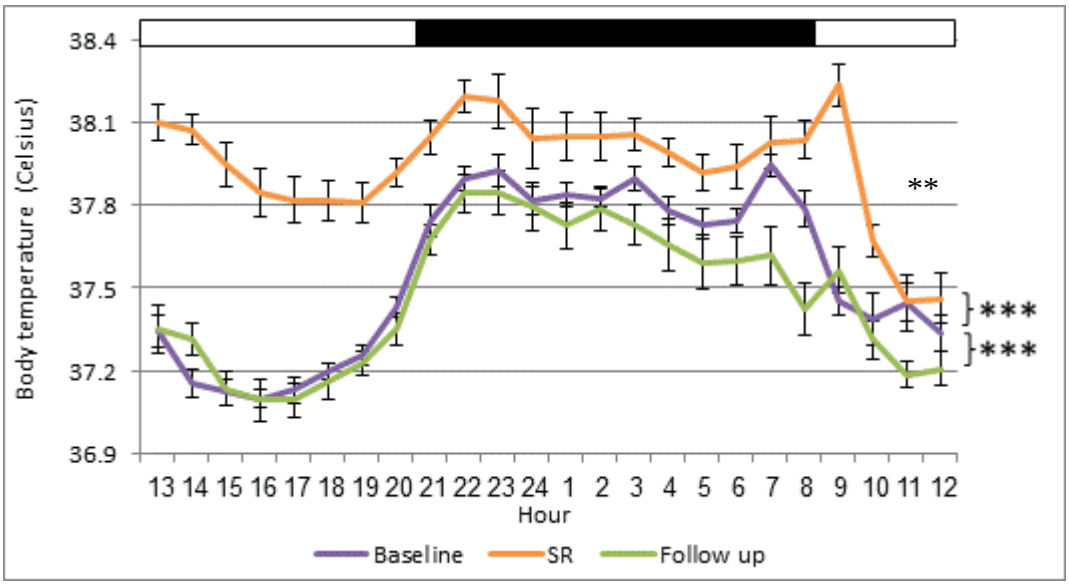

Figure 6: Average circadian body temperature within the SR group: SR animals' body temperature was higher than baseline during SR days. SR animals' body temperature was lower than baseline during follow up, only during the dark phase. The horizontal white and black bars represent light and dark, respectively.
[N: SR - 12 (*** significant difference between baseline and SR days or follow-up, p<.001)].

The behavioral effects of exposure to the Sleep restriction protocol

Object location recognition: Throughout the days of the habituation, no significant differences were found between groups in the total distance moved, distance moved in the center, duration in the center or the number of enters to the center, [$t_{(24)}$= 1.11, NS; $t_{(24)}$= 0.78, NS; $t_{(24)}$= 0.93, NS; $t_{(24)}$= 0.34, NS], respectively, using t-test. Apparently there was no significant difference in the activity level or anxiety level between groups as measured by the open field.

In the learning phase, no significant differences between the exploration times of objects were found in both groups, using Sign test for related samples [z=0, NS]. In the test phase, the preference for the new location was calculated with a ratio between exploration time of the new location divided by the total objects exploration. Average ratio of both groups was above 0.5 showing some preference for the new location (0.58 for the control group (n=10) and 0.66 for the SR group (n=10)), but there was no significant difference between them, using t-test [t(20)= 1.12, NS]. No significant difference between groups was found in their location recognition memory.

Two-way shuttle avoidance learning: as depicted in figure 7A, SR group had a significantly higher average percentage of avoidance on the second day of the test comparing to the control group, using the Mann-Whitney U test [U= 130.5, p<0.05]. In addition, as depicted in figure 7B, Repeated measures ANOVA indicated a significant main effect for group on average avoidance times per

block (each 15 trials) [$F_{(1,24)}$= 8.12, p<0.01]. Apparently the SR group had a better avoidance learning curve compared to the control group.

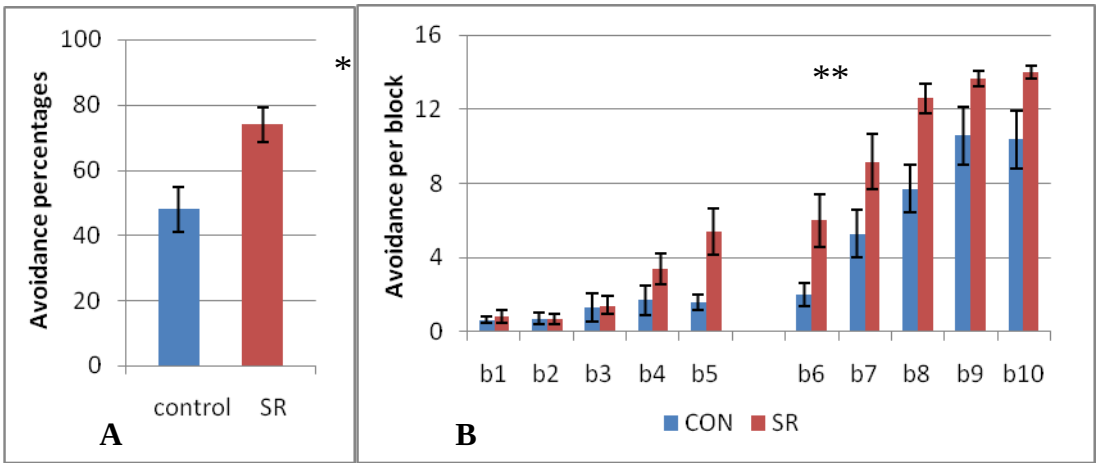

Figure 7: Average avoidance percentages on the second day [A] and avoidance learning curve [B] in the TWSA: SR group had a significantly higher average percentage of avoidance on the second day of the test comparing to the control group. Additionally, although both groups had learned gradually to avoid the shock, the SR group had a significantly better learning curve.

[N: control - 14, SR - 12. (* significant difference between groups, p<.05, ** significant difference between groups, p<.01)].

Conclusions

The aim of task #1 was to verify that in our hands the SR protocol indeed works and that it challenges animals physiology in some way. In addition, this stage was required in order to verify that the SR equipment (motorized wheels) and the DSI telemetry system can be used together. Both aims were achieved. The Meerlo et al., (2002, 2008) protocol was found to be effective and to affect activity and body temperature during the SR days and in the follow up days. The effects were not dramatic but this was expected since we have deliberately selected a mild protocol of SR and not of sleep deprivation (SR animals had four hours of sleep each day without disturbance). This protocol was selected because of it was considered potentially more relevant to the conditions of deployed soldiers.

Interestingly, immediately following the SR exposure, animals were faster to learn the two-way shuttle avoidance task, despite the fact that this is a learning-under-stress task. One possible explanation is that these animals are at a higher level of alert. If this assumption holds than it is expected that additional stress would more easily drive them beyond the effective level of alertness and will more readily lead to impaired performance. This possibility is to be examined.

Task 3: **Behavioral and neurobiological investigation of the impact of the under-water trauma (UWT) with or without a reminder cue –**

3.1 The effects of a reminder cue of an underwater trauma on behavior and memory-related mechanisms in the rat hippocampus

Introduction

Experiencing a life-threatening event is essential for the development of post-traumatic stress disorder (PTSD - hereafter). Many studies that have examined the consequences of an exposure to stressfull events in adulthood were able to demonstrate behavioral and physiological alterations in response to stress, depending on the stress protocol that was used (for a review see Armario et al. 2008).

One stress model is the underwater trauma (UWT- hereafter) (Richter- Levin, 1998), in which the animal is restrained under the water for 30-45 seconds. It has been already shown that exposure of rats to UWT results in increase in anxiety like behaviors (Cohen et al. 2004; Richter-Levin 1998), context-specific spatial memory deficits (Richter-Levin 1998; Wang et al. 2000) and an impairment in LTP induction in the hippocampus immidiately after exposure to the UWT, that was correlated with memory deficits found in the Morris water maze (Wang et al. 2000). Apart from testing the effects of the exposure to the UWT itself, Cohen and colleagues (2008), have demonstrated that when a reminder cue associated with UWT is presented in a new context, it triggers a fear response (freezing behavior), while a reminder cue associated with other stressors (i.e. elevated platform or restraint) doesn't elicit this type of response (Cohen et al. 2008).

Intrusive re-experiencing is a core symptom of PTSD that can take various forms, including intrusive images, flashbacks, nightmares, distress and physiological reactions. It has been suggested that such episodes might be triggered when confronting with reminder cues (i.e. sounds, smells, locations, and activities) associated with the traumatic event (diagnostic criterion B; American-Psychiatric-Association 1994; Bower and Sivers 1998; Elzinga and Bremner 2002). The fact that, unlike other stressors (i.e. elevated platform or restraint), reminder cue of UWT elicited fear response in a new context (Cohen et al. 2008) suggests that UWT can also act as a stress model to study the intrusive re-experiencing phenomena in PTSD.

The effects of stressful experience on behavior can be tested by using behavioral assessments (e.g. elevated plus maze and open field) that aim to evaluate the emotional state of the animal (Avital et al. 2006; Jacobson-Pick and Richter-Levin 2010). In addition to the behavioral effects, stress has also been found to affect different measures of synaptic plasticity (Kim et al, 2006).

Electrophysiological studies on LTP in the hippocampus dentate gyrus (DG - hereafter) have shown that, depending on the stress paradigm being used, stress can impair (Akirav and Richter-Levin 1999; Shors and Dryver 1994), enhance (Kavushansky et al. 2006) or leave unaffected (Bramham et al. 1998; Gerges et al. 2001).

Although LTP is a widely accepted model of learning and memory, there is a continuing debate over its validity, and its behavioral correlates (Mozzachiodi and Byrne, 2010). Another level of processing that might be relevant to memory formation is local circuit activity. When targeting the local circuit level, the focus is on interactions between local, mostly inhibitory GABAergic neurons and pyramidal or granular principle cells in the hippocampus and cortex (Freund and Antal 1988; Freund and Buzsaki 1996). Previous work in our lab has already shown that stressful experience may lead to alterations in local circuit activity and plasticity (Yarom et al. 2008).

In the current study we aimed to examine the impact of a reminder cue of an UWT on anxiety and fear responses but also on memory-related mechanisms in the hippocampus. Besides testing the effects of exposure to a reminder cue on the ability to induce LTP, alterations in local circuit activity in the dentate gyrus following exposure to the reminder cue were also be addressed.

Methods

Animals

Male Sprague Dawley rats (~60 days old, 250-350 g) were used for the experiments. Animals were housed in groups of 4, at 22 ± 2°C under 12-h light/dark cycle. Water and food were available ad libitum.

Experimental groups

Following acclimation all rats were randomly assigned to one of the following experimental conditions:

1. Underwater trauma + reminder (UWT+R) – Rats exposed to 'swim trials', 'underwater trauma' and a 'reminder cue'.
2. Underwater trauma (UWT) – Rats exposed to 'swim trials' and 'underwater trauma'.
3. Control + reminder (Control+R) – Rats exposed to 'swim trials' and a 'reminder cue'.
4. Control (Control) – Rats exposed to 'fswim trials'.
5. Naïve – Rats that were exposed to neither the 'swim trials', 'underwater trauma stress' nor the 'reminder'.

Experimental design

Figure 1 summarizes the experimental protocols used in this work. Following delivery and an acclimation period of five days, rats were randomly assigned to 3 groups. The rats from the first two groups, each containing 16 rats, were exposed to swim trials (1 min. of swimming per day), for five consecutive days. On the 6th day, the rats from the first group were exposed to under water trauma (UWT) while the rats from the second group were exposed to an additional session of 1 min. of swim. On the 7th day, 24 hrs after the last exposure (i.e. UWT or 1 minute of swim), 16 rats (8 rats from each of the first two groups) were exposed to a reminder, thus creating four experimental groups: UWT without reminder (UWT), UWT with reminder (UWT+R), swim only (Control) and swim with reminder (Control+R). The original third group of 8 rats, the 'naïve' rats, was not exposed to any of the mentioned treatments (swim, UWT and reminder).

Two sets of experiments were conducted using the same experimental procedure as described above. The first experiment was conducted in order to validate/investigate the behavioral effects of the exposure to the reminder of the 'UWT' stress. After establishing this step, an additional experiment was conducted in order to assess the electrophysiological effects of the exposure to the reminder of the 'UWT' stress. Different subsets of animals were used in each one of the above experiments.

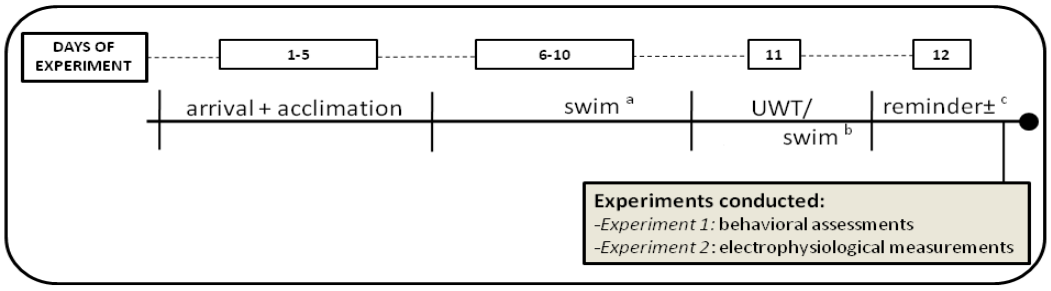

Figure 1. Experimental design

a. 'UWT' and 'Control rats' were exposed to five consecutive days of swim trials, 'naïve rats' remained in their home cage.

b. 'UWT' rats' were exposed to UWT stress, 'Control rats' were exposed to an additional session of swim and 'naïve rats' remained in their home cage.

c. 'UWT' and 'Control rats' assigned to the reminder condition were exposed to the reminder cue while 'UWT' and 'Control rats' of the no reminder condition remained in their home cage. 'Naïve rats' remained in their home cage.

Behavioral procedures

Swim trials

After 1 min. of habituation to the room, rats were subjected to a swim procedure by placing them in a plastic tank (diameter 40 cm, height 45 cm) that contained 30 cm height of water at 22± °C for 1 min. Animals were exposed to a single swim trial per day for 5 consecutive days.

Underwater trauma protocol

The underwater trauma was carried out by placing a rat in a plastic tank (the same one that was used in the five days of swim). After 1 min. habituation to the room, rats were given 30 sec. of free swimming and then were held under water for additional 30 sec, using a special metal net (20X20X15cm.), (adapted from Wang et al., 2000).

Reminder

The reminder was designed as a shorter version of the swim procedure. After 1 min. habituation to the room, rats were placed in an empty plastic tank (the same one that was used for the swim trials and for the UWT) for 30 sec. The exposure to the reminder cue was conducted 24 hrs. after the last exposure (i.e. UWT or swim).

The swim, underwater trauma and the reminder cue presentations were carried out between 9:00 to 15:00.

Behavioral assessments

After the exposure to the reminder, 'UWT' and Control rats' were returned to their home cages for 30 min. and then they were taken to the behavioral tests. Naïve rats were taken to the behavioral tests straight from their home cage.

Elevated Plus Maze test

The elevated plus maze test was carried out according to methods described previously (Pellow et al., 1985). Briefly, the maze is placed 50 cm above the floor and consists of two open arms and two closed arms (with 30cm high Plexiglas walls and no roof), arranged in a way that similar arms are opposite to each other. Before each test, the animal was allowed to habituate to the room for 5 min., after which the animal was placed in the center of the maze, facing an open arm, and was allowed to explore the arena for 5 min., while its behavior was videotaped. Each of the recorded sessions was blindly analyzed offline by a different experimenter.

Open Field test

The open field test was carried out according to methods described previously (Avital and Richter-Levin, 2005). Briefly, the open field test consists of a square Plexiglas box (50×50×38 cm) placed inside a dimly red-lit ventilated sound-attenuated cupboard. The walls are painted black, the floor is white and divided by 0.3cm-wide black lines into 25 equal squares of 10×10 cm each. Every Open field test starts with 5 min. habituation to the room, followed by placing the rat at the corner of the open field facing the wall. The rat was allowed to explore the novel environment for 5 min while its behavior was videotaped. Each of the recorded sessions was later blindly analyzed by a different experimenter.

Electrophysiology

The anesthesia and electrophysiological procedures were performed in strict accordance with University of Haifa regulations and National Institute of Health (NIH) guidelines.

Male Sprague Dawley rats (250-350 g) were anesthetized (with 40% urethane and 5% chloralhydrate in saline, 0.5 ml/100g, intraperitoneally [i.p.]) and placed in a stereotaxic frame with body temperature maintained at 37±0.5°C by a regulated heating pad during the course of the experiments.

After fixing the head of the anasthetized animal in the stereotaxic frame, small holes were drilled in the skull to allow insertion of electrodes into the brain. A recording microelectrode (glass, tip diameter 2-5 µm, filled with 2M NaCl, resistance 1-4 MΩ) was placed in the dentate gyrus (coordinates: 4 mm posterior to bregma, 2.5 mm lateral to midline). A bipolar 125 µm stimulating electrode was placed in the ipsilateral angular bundle to stimulate the perforant path (coordinates: 8 mm posterior to bregma, 4 mm lateral to midline). The depth of the electrodes was adjusted to maximize the size of the evoked positive-going EPSP recorded in the hilus of the dentate gyrus. DG field potentials were evoked by single pulse stimulation delivered to the PP, (100 msec. rectangular monophasic pulses, model of stimulator), amplified (×100) by AM systems amplifier (model 1800) and stored on a PC hard drive for off-line analysis (CED spike2 software) .

Electrophysiological protocols:

After positioning the electrodes in the brain, the rat was let to recover for 20 min, before the beginning of the experiment. Baseline recordings were made for 30 min. The test stimuli for baseline recordings were monopolar pulses of 100 µsec duration each, with stimulation intensity of ~1.5 mA and frequency of 0.1Hz. The test stimuli were adjusted to yield a population spike (PS- hereafter) of 20-40% of the maximal pre-tetanic field potential amplitude. The amplitude of the PS and the slope of the EPSP were measured offline from averages of 10 successive responses to a given stimulation intensity, applied at 0.1 Hz.

LTP Induction

Theta burst stimulation (TBS- hereafter) of the PP was used to induce LTP. The TBS protocol included 3 sets of 10 trains each, where each train consisted of 10 pulses at 100 Hz, at baseline stimulation intensity, inter-train interval of 200 ms and 1 min. interval between sets. The LTP was measured as the difference in EPSP slope before and 30 min. after TBS. LTP was defined as an increase of at least 20% in the EPSP slope of the evoked potentials 30 min. after TBS stimulation. PS amplitude units of measurements are presented in mV, or as percentage of the baseline response. EPSP slope units of measurements are presented in mV/sec, or as percentage of the baseline response.

Frequency-dependent inhibition (FDI)

To determine FDI, 10 baseline pulses were delivered to the PP at 0.1 Hz, followed by 10 pulses delivered at 1 Hz. The PS amplitude of the 10 responses to PP stimulation at 0.1 Hz were averaged and compared to the 10 responses to PP stimulation at 1 Hz. The averaged response to the stimulation delivered at 0.1 Hz was set as 100%, and the averaged responses at 1 Hz were expressed as the percent change of the response at 0.1 Hz. (FDI index)

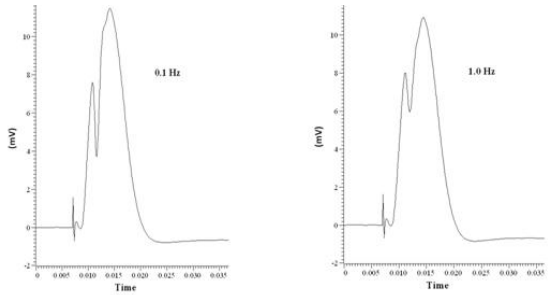

Figure 2. **Frequency-dependent inhibition (FDI):**
Representative field potential response of DG granule cells to stimulation of the PP at 0.1 Hz (left) or at 1.0 Hz (right). Altering the frequency of stimulation from 0.1 Hz to 1.0 Hz resulted in suppression of the PS amplitude. Time= seconds.

Paired Pulse Inhibition (PPI)

Paired-pulse inhibition (PPI) was measured by applying five pairs of two constant stimuli to the PP at inter-stimuli interval of 15 msec.

The PS amplitude of the five responses to the first stimuli delivered to the PP were averaged and compared to the five responses to the second stimuli delivered to the PP 15 msec. after the first stimuli.

The averaged response to the first stimulation delivered to the PP was set as 100%, and the net mediated inhibition measured by paired pulse protocol was expressed as the percent change of the PS amplitude of the second response with regard to the PS size of the first one. (PPI index)

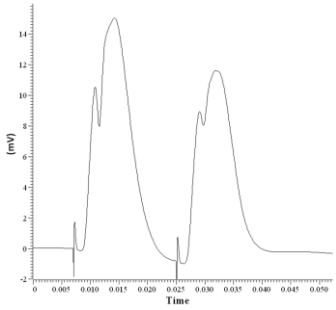

Figure 3. **paired-pulse inhibition**: Representative field potential responses to double stimulation of the PP at 15 msec interval. Applying two constant stimuli to the PP at inter-stimuli interval of 15 msec resulted in suppression of the PS amplitude of the second response. Time= seconds.

Electrophysiology recording protocol

After placing the rat in a stereotaxic frame, positioning the electrodes and 20 min. of recovery period, offline measurements of input-output curve response were made to determine the stimulation intensity for baseline response. The following recording protocol was then conducted; first baseline (1^{st} baseline- hereafter) was recorded for 30 min, immediately followed by the local circuit protocols of FDI and PPI (in that order). After the local circuit protocols, a second baseline (2^{nd} baseline- hereafter) was recorded for another 20 min. to serve as pre TBS values. This was followed by a TBS and LTP measurement for additional 30 min.

Statistical Analysis

Differences were evaluated using one-way or repeated measures analysis of variance (ANOVA). All post hoc comparisons were made using the least significant difference multiple comparison tests.

Results

Two experiments were designed to test the effects of an exposure to a reminder cue of the 'UWT'. Experiment I assessed the behavioral effects of the exposure, while experiment II tested the effects of the exposure to a reminder cue on local circuit activity and on LTP induction and in the hippocampus DG.

Experiment I – The behavioral effects of exposure to a reminder cue of UWT

Open field test: as shown in figure 4, the rats that were exposed to the reminder cue spent less time in the center of the open field (figure 4A, $F_{(4,62)}= 7.99$, $p<0.01$) and were less active (figure 4B, $F_{(4,62)}= 9.60$, $p<0.01$, one way ANOVA). Further Post hoc comparisons indicated that UWT and UWT(+) rats spent less time in the center of the arena and were less active, compared to naïve rats. Moreover, while UWT rats did not differ from Control and Control(+) in time spent and/or level of activity in the center of the arena, UWT(+) rats spent less time and were less active in the center of the arena compared to Control, Control(+) and UWT rats, ($p<0.05$).

Elevated plus maze test: as depicted in figures 5-AB, One way ANOVA indicated a significant main effect for the exposure on time spent in the open arms, on number of entries to the open arms and on activity in the open arms [$F_{(4,61)}=3.74$, $p<0.01$; $F_{(4,61)}= 6.08$, $p<0.01$], respectively. Further Post hoc comparisons indicated that UWT(+) rats entered less frequently and spent less time in the open arms of the elevated plus maze compared to all the other groups, ($p<0.05$).

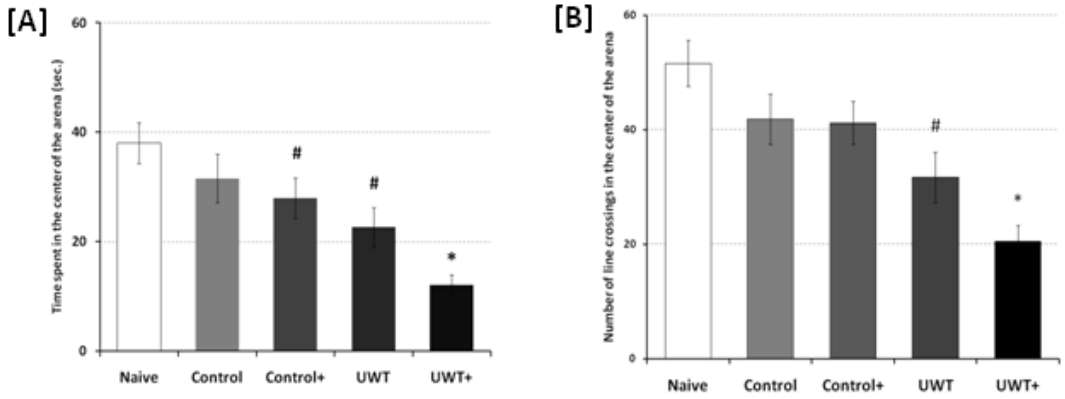

Figure 4: Time (sec) (A) and activity (B) in the Open field test**:** exposure to a reminder cue following an axposure to 'UWT', reduced both time and activity in the center of the arena, compared to all other groups. An exposure to 'UWT' whithout an exposure to a reminder cue also reduced time and activity in the center of the arena but only compeard to naïve group. [N: naïve-14, control -12, control (+)-15, 'UWT'-12, 'UWT'(+)-14. (*significant difference from all groups, p<.05, # significant difference from naïve group, p<.05].

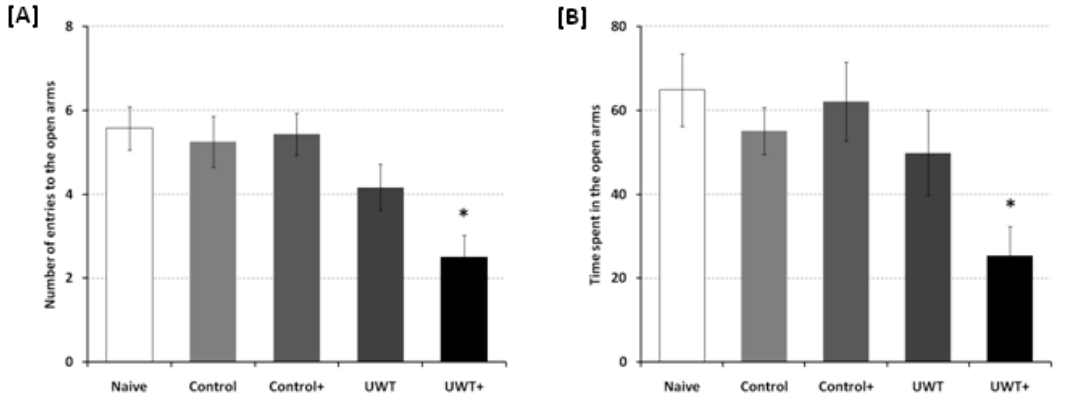

Figure 5-AB: Time (sec) spent (A) and number of entries in the open arms of the elevated plus maze (B)**:** exposure to a reminder cue following an axposure to 'UWT' reduced both time and number of entries to the open arms, compared to all other groups. [N: naïve-14, control-12, control(+)-14, 'UWT'-12, 'UWT'(+)-14. (*significant difference from all groups, p<.05, # significant difference from naïve group, p<.05].

Experiment II – The electrophysiological effects of exposure to a reminder cue of UWT

Exposure to a reminder cue of UWT does not affect baseline responses in PP-DG pathway

As depicted in table 1, One-way ANOVA did not reveal any significant difference in stimulus intensities applied to all groups [$F_{(4,35)}$ = .156, *n.s.*]. In addition, Comparison between the groups using ANOVA with repeated measures for the time points before the application of local circuit protocols ('1st baseline' - hereafter) did not reveal any significant difference in baseline responses. No significant differences were found for PS amplitude or EPSP slope [F(5,31) =1.297, *n.s.*; F(20,118) = 0.816, *n.s.*], respectively. The average of the PS amplitude and EPSP slope during baseline recording is presented in Table 2.

Table 1. The stimulus intensities in the different groups

Group	Intensity (mA)
Naïve	1.51 ± 0.17
Control	1.52 ± 0.17
Control (+)	1.60 ± 0.21
UWT	1.64 ± 0.21
UWT (+)	1.47 ± 0.11

Note. Table 1 summarizes the intensity applied to the different groups and shows that similar stimulus intensities were applied to all the groups.

Table 2. 1st baseline population spike amplitude and EPSP slope in DG in the different groups

Group	PS amplitude (mV)	EPSP slope (mV/sec.)
Naïve	3.66 ± 0.53	5915.51 ± 351.57
Control	2.47 ± 0.26	6632.98 ± 479.15
Control (+)	4.43 ± 0.42	5798.73 ± 347.97
UWT	2.55 ± 0.22	6337.48 ± 646.02
UWT (+)	3.77 ± 0.39	5941.40 ± 335.92

Note. Table 2 summarizes the averaged baseline PS amplitude and the averaged EPSP slope of the different groups. The groups did not differ in their PS amplitude or in their EPSP slope.

The effects of exposure to a reminder cue on local circuit activity in the DG

Paired- pulse inhibition

As depicted in figure 6-A, upon delivering paired pulse stimulation to the PP, One way ANOVA [$F_{(4,35)}= 5.54$, $p<0.01$], revealed a significant reduction of the PS amplitude of the response to the second stimuli to the PP compared to the response to the first stimuli at a 15msec interval in all groups (indicated by the PPI index). Further Post hoc comparisons indicated that compared to all other groups (i.e. Naïve, Control and Control (+)), both UWT and UWT(+) rats exhibited stronger inhibition of PS amplitude as was expressed by the low PPI index, ($p<0.05$).

Frequency- dependent inhibition

As depicted in figure 6-B, upon altering the frequency of the stimulation to the PP from 0.1Hz to 1.0 Hz, One way ANOVA [$F_{(4,35)}= 4.59$, $p<0.01$] indicated a significant reduction of the PS amplitude in all groups, compared to its amplitude when stimulating at 0.1 Hz (indicated by the FDI

index). Further Post hoc comparisons indicated that UWT(+) rats showed stronger inhibition on PS amplitude as was expressed by the low FDI index compared to all the other groups ($p<0.05$).

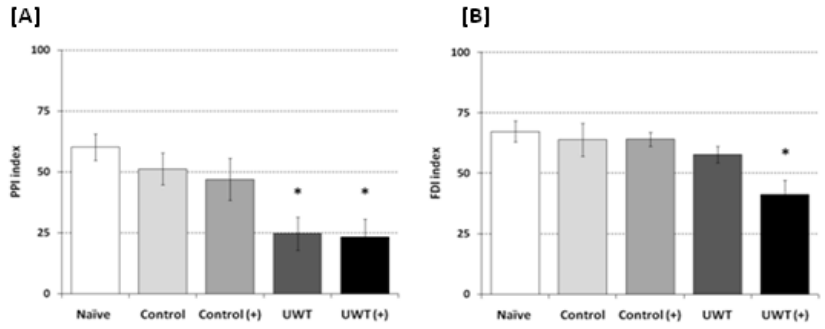

Figure 6-AB: [A] Paired pulse inhibition; upon delivering double stimulation of the PP at 15 msec interval, both UWT groups (N: UWT – 8 UWT (+) – 8) showed stronger inhibition of PS amplitude as was expressed by the low PPI index, compared to all other groups (N: naïve – 8, control – 8, control (+) – 8; * - significantly different from other groups, p= 0.05). **[B] Frequency dependent inhibition**; upon altering the frequency of the stimulation to the PP from 0.1Hz to 1.0 Hz, UWT(+) rats (n=8) showed stronger inhibition on PS amplitude as was expressed by the low FDI index, compared to all other groups (N: naïve – 8, control – 8, control (+) – 8, UWT – 8; * - significantly different from other groups, p= 0.05).

The effect of exposure to a reminder cue of UWT on LTP induction in the DG

Comparison between the groups using ANOVA with repeated measures for time points before the application of TBS ('2nd baseline' - hereafter) did not revealed any significant difference in PS amplitude or in EPSP slope [$F(3,33) = 0.556$, *n.s.*; $F(12,95) = 1.498$, *n.s.*, respectively]. The averages of the PS amplitude and EPSP slope during baseline recording are presented in Table 3.

Table 3. 2nd baseline population spike amplitude and EPSP slope in DG in the different groups

Group	PS amplitude (mV)	EPSP slope (mV/sec.)
Naïve	3.66 ± 0.53	6367.18 ± 446.83
Control (-)	2.47 ± 0.26	6688.14 ± 479.91
Control (+)	4.43 ± 0.42	6171.16 ± 405.54
UWT (-)	2.55 ± 0.22	6468.68 ± 666.39
UWT (+)	3.77 ± 0.39	5717.58 ± 373.68

Note. Table XX summarizes the average baseline PS amplitude and the average EPSP slope of the different groups and shows that the groups did not differ in their amplitude or in their EPSP slope.

As depicted in figure 7-A**,** comparison of PS amplitude between the different groups using ANOVA with repeated measures for time points after the application of TBS did not reveal any significant

effect for post-TBS time [F(5,31) =0.706, *n.s.*] or for the interaction time × exposure [F(20,118) = 1.522, *n.s.*].

As depicted in figure 7-B**,** comparison of EPSP slope potentiation using ANOVA with repeated measures for time points after the application of TBS revealed a significant effect of post-TBS time [F(5,31) = 10.346, ($p<0.01$)] and for the interaction time × exposure to [F(20,118) = 2.042, ($p<0.01$)]. Post hoc comparisons indicated that UWT(+) rats exhibited an impairment in LTP induction, compared to all the other groups ($p<0.05$).

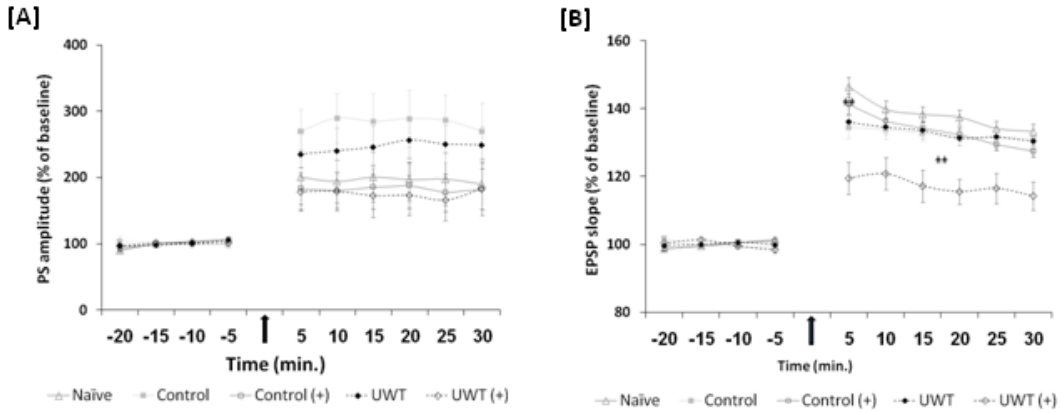

Figure 7-AB: [A] Application of TBS significantly increased the level of potentiation of the PS amplitude in all groups (N: naïve – 8, control – 8, control (+) – 8, UWT – 8, UWT (+) – 8), no significant differences were found between the groups. **[B]** Application of TBS significantly increased the level of potentiation of the EPSP slope in all groups (N: naïve – 8, control – 8, control (+) – 8, UWT – 8, UWT (+) – 8). Furthermore, UWT (+) rats showed an impairment in the ability to induce LTP, compared to all other groups (** - significantly different from other groups, p= 0.001).

Reminder cue – maps of brain activation as measured by activation of ERK II

Tissue that was collected from rats exposed to the protocols above was used to initiate the examination of maps of brain regions activation following the exposure to a reminder cue of the UWT.

In an initial study we have used the activation by phosphorylation) of the MAP Kinase Cascade enzyme ERKII. We have examined the activation in the dorsal DG (the area in which electrophysiology was measured), as well as the basolateral amygdala (BLA), since previous work in our lab has indicated that emotional and traumatic experiences may influence hippocampal activity by activating the amygdala (Tsoory et al, 2008).

Methodology:

Immunoblot analysis

30 minutes following the exposure to the reminder rats were decapitated, their brains were removed and quick freezed (using dry ice powder). Hippocampus dorsal DG and BLA brain

regions were harvested. The tissues were collected into 1.5 ml Eppendorf tubes, immediately frozen in liquid nitrogen and stored at –80°C until further use. Tissues where homogenized in a glass Teflon homogenizer in 180-700 µl of ice-cold Urea Lysis Buffer [(1mM EDTA (Fluka), 0.5% Triton X (SIGMA), 6M Urea (SIGMA), 100µM PMSF (SIGMA] with freshly added protease and phosphotase inhibitors [0.1 mM sodium orthovanadate, 1 lg/ml leupeptine, 1.6 lg/ml aprotinin, 5 mM NaF, and 1 lg/ml protease inhibitor cocktail P2714 (from Sigma, St. Louis, MO)] and incubated at 100°C for 5 min. Samples of 10 µg (amount loaded was calibrated in a pilot study) were loaded in each lane of the 10 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Following 1 hour semi-dry transfer (60mA per membrane) onto a 0.45µm nitrocellulose membrane the lanes were compared for gross protein homogeneity loading by Ponceau staining (SIGMA). Blots were blocked using 3% BSA in Tris-Buffered Saline Tween-20 (TBST: 0.9% w/v NaCl, 0.05% v/v Tween-20 and 100 mM Tris- HCl, pH 7.6) incubation for 45 min at room temperature (RT). The membranes were incubated overnight on a shaker with first antibody in TBST at 4ºC. The next day excess of first antibody was washed 3 times for 10 min with TBST. Secondary α-rabbit antibody incubation conducted for 1 hour at RT. The membranes were washed 3 times, 10 min each, in TBST before development, with EZ-ECL chemiluminescence light reaction (Amersham, Piscataway, NJ) using the CCD camera (XRS BioRad).

Immunoblot Reagents:

In order to determine cell signaling processes, the following reagents, from Cell Signaling (Beverly, MA), were used: α-ERK1/2 (p44/42 MAP kinase) antibody and α-p-ERK2 (phospho-p44/42 MAP kinase; Thr202/Tyr204) antibody (Both 1:1,000), rabbit polyclonal (1:10,000).

Quantification

Densitometric analysis of ERK2 immunoreactivity was conducted in Quantity One 1-D Analysis software. Each sample was measured relative to the background, and phosphorylation levels were calculated as the optical density (OD) ratio between the phosphorylated (phospho-ERK2) and the nonphosphorylated (ERK2) forms of the protein. The results were normalized to the Naïve group values.

Results

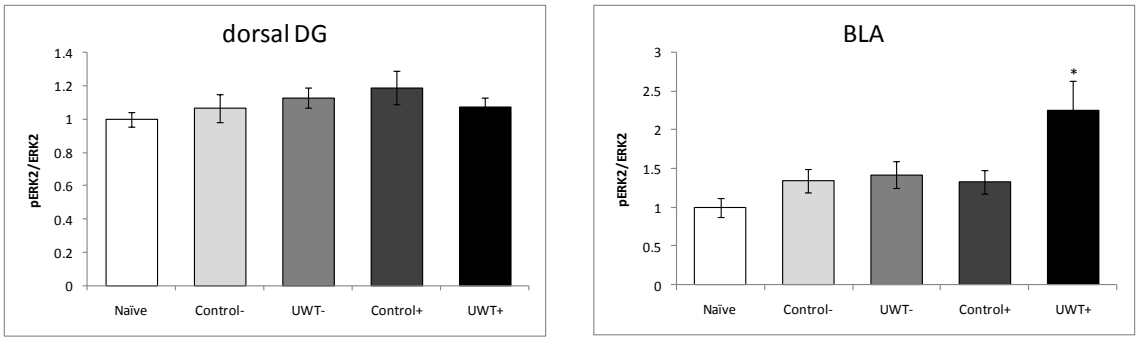

Figure 8: A significant activation of the BLA, as indicated by the activation of ERKII, was found only in the group that was exposed to the UWT and to the reminder cue before tissue collection. No such activation was found in the DG, suggesting that some of the effects of the reminder cue on hippocampal electrophysiology may be mediated by activation of the amygdala.

Discussion

In this project we have further verified the UWT as a model for exposure to an acute stressful event. Twenty four hours following the exposure to UWT animals still exhibited increased anxiety in the open field and enhanced Paired pulse Inhibition in the DG. However, the findings demonstrate in addition the impact of the reminder cue. The UWT(+) group exhibited higher levels of anxiety, that in the elevated Plus maze were significantly higher even from the UWT group, and in addition exhibited impaired LTP and enhanced Frequency Dependent Inhibition, effects that were not found in the UWT group.

Furthermore, we have started to examine the neurobiological mechanisms associated with the effects of the reminder cue. Initial findings suggest that the activation of the amygdala (or more specifically, of the BLA) may be involved in mediating some of the effects of the reminder cue.

While the results are a clear demonstration of the impact of a reminder cue, there are two aspects to it that we felt deserving further evaluation:

First, in this experiment animals were tested 24 hrs following the exposure to the UWT. While the impact of the reminder cue could clearly be demonstrated, 24 hrs could still be considered to be within the period of acute stress responses, and not necessarily related to PTSD. We wanted to examine whether such effects could be found also much later, weeks after the exposure to the initial trauma.

Second, here the reminder cue was exposing the animal again to swim. Although exposing it to swim is not re-exposure to the UWT, the reminder cue was in a way returning to the trauma scene,

not just being exposed to a reminder of it. We thought it would be important to examine whether exposure to a reminder cue, out of the immediate context of the trauma, would have similar effects. The next stage of the project is thus designed to address these questions.

Task 4: **The impact of sleep restriction on the outcome of exposure to UWT-**

Relevant to the human condition, there is individual variability of the intensity and severity of symptoms. We are examining the assumption that sleep restriction increases the risk of developing long lasting pathological symptoms following an exposure to a traumatic event. This task is currently underway.

Aims

Research lacks studies concerning the interacting risk factors for PTSD. In the proposed study we aim to examine the impact of a hypothesized risk factor that is proximal to the traumatic event – sleep restriction (SR) immediately prior to the event - on the development of PTSD-related symptoms later on. Later on we will investigate the interacting effect of SR with another risk factor that is rather distal to the traumatic event – a history of stress in juvenility. Juvenile stress (JS) may result in alterations in sleep patterns and biorhythms that could mediate its aversive impact on coping with stress in adulthood. Yet, additional possibility is that JS will induce other alterations that will render the individual more vulnerable to sleep restriction effects later in life.

Additionally, we would like to examine the involvement of positive and negative affect systems (i.e. the 'FEAR' and 'SEEKING' versus the 'PANIC' systems (Panksepp, 1998)) in the development of depressive and/or anxiety-like symptoms in the consequences of exposure to UWT on the background of exposure to JS and SR. To this end we will study the involvement of GABA and related proteins in specific brain areas related to the core emotional systems.

Hypotheses

We hypothesize that UWT will have moderate aversive consequences in sleep restricted rats. Furthermore, we speculate that UWT will have a far more aversive outcome in sleep restricted rats that have previously been exposed to JS. Specifically these are the hypotheses:

1. Exposing animals to the UWT will result in lasting alterations at the behavioral, and biochemical levels. These will be found in most of the animals 24 hrs following the exposure to the trauma, but only in some of the animals 4 weeks later.

2. An exposure to SR protocol in the days prior to the exposure to the UWT will result in an increase in the number of animals demonstrating PTSD-related symptom 4 weeks after the trauma.

3. Prior exposure to JS will significantly exacerbate the long-term impact of SR. A larger number of animals will exhibit more sever PTSD-related symptoms even 4 weeks after the UWT, at all levels that will be tested.

Within the framework of task 4 we will focus on the impact of SR on the outcome of UWT. Task 6 that will begin in parallel and will be devoted to the outcome of interaction of distal and proximal risk factors on the outcome of the UWT.

Experimental Procedure.

Rats are randomly allocated to experimental groups, detailed below. All through the experiment starting at the age of 27 days, saccharine preference is evaluated (27-102 PNDs) among all rats, as an index for the animals' anhedonia. At 45 PND they are implanted with a radio transmitter (model TA10TA-F40; DSI., St Paul, USA), that enables monitoring of body temperature and activity. After surgery, animals have 7 days to recover. At 53 PND telemetry recordings begin, starting with 7 days of baseline (53-102 PNDs in total). Before the SR protocol the rats are habituated to the SR apparatus by placing them in the wheels for 1 h on 3 successive days (57-59 PNDs; Slowly or voluntary rotating wheels, according to the experimental group; Home cage control group is not habituated). At PND 60, part of the rats are exposed to SR for 8 days. Another part of the rats are exposed to the control procedure of SR, while the remaining rats stay at their home cages. At 74 PND half of the rats that were exposed to SR or SRcont, are exposed to the UWT that serves as an 'Adult-Stress'. Two hours following the UWT, blood samples are taken from all rats' tail and analyzed for corticosterone. An assessment of the animals' behavioral performances is conducted using Behavioral battery A at PND 75, following the exposure to the 'Adult-stress'. In order to evaluate the chronic response to an 'adult-stress', an additional assessment of the animals' behavioral

performances is conducted using Behavioral battery B, 4 weeks following the 'Adult-stress', at PND 102. Timing of procedures is illustrated in Figure 1.

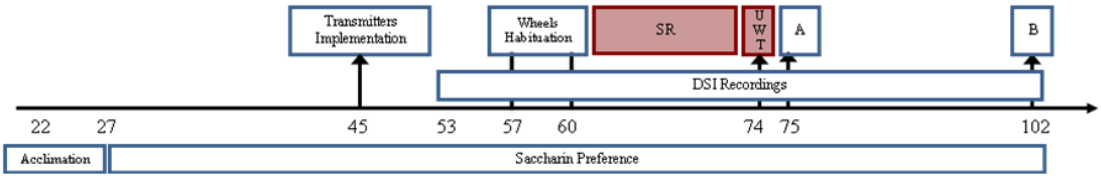

Figure 1: Schematic illustration of the experimental procedure of task 3. For details please see text.

Experimental groups.

Rats are randomly assigned to one of the following SR and/or stress exposure experimental conditions:

1. Sleep restriction and adulthood stress (SR+AS) – Rats are exposed to SR and UWT.
2. Sleep restriction (SR+A0) – Rats that are only exposed to SR but not to UWT.
3. Sleep restriction control and adulthood stress (SR_{cont}+AS) – Rats that are exposed to the control procedure for SR and to UWT.
4. Sleep restriction control (SR_{cont}+A0) – Rats that are only exposed to the control procedure of SR.
5. Home cage control (SR0+A0) – Rats that are exposed neither to the UWT nor to SR.

Saccharine preference. Is conducted over 76 days (27 – 102 PNDs), for all rats as a measure of 'anhedonia'. Rats are continuously offered a choice between two calibrated burettes: one filled with the 0.606% saccharin solution (Sac) and the other filled with water (Wat). The amount of liquid intake is measured in milliliters every other day, and the burettes' place are switched to avoid learning. Saccharin preference is calculated as the amount of saccharin intake (ml), divided by the total intake of water (ml) and saccharin every other day.

Saccharin preference = Sac. (ml)/ [Sac. (ml) + Wat. (ml)]

Behavioral battery A. This behavioral assessment battery consists of the open-field test, elevated plus maze test, a social interaction test and a social recognition test:

- *The open field test.* The open field consists of a wooden box 90.0cm × 90.0cm × 38.0cm, positioned in a dimly-lit room. The walls are painted black; the floor is white and divided by 0.5 cm wide black lines into 25 squares 17.0 cm×17.0 cm. Following 5 min habituation period for the testing room, rats will be placed at a corner of the open field for 5 min of free exploration. The number of line crossings and the time spent in the central and the peripheral areas will be manually recorded. The total line crossings will represent the activity level of the rat. The ratio between line crossings in the peripheral area and the total line crossings, and the relative time spent, will be considered a measure of anxiety.
- *The elevated plus maze test.* This maze consists of a plus-shaped platform with two open arms and two closed arms surrounded by 38-cm high opaque walls on three sides, with arms of the same type located opposite each other (File, 1993). Each rat will be placed on the central-platform facing an open arm and will be allowed to explore the maze for 5 min, following 5 min habituation period for the testing room. Arm entry will be defined as entering an arm with all four paws. The following measures will be used: durations in open and closed arms, and on the central-platform; open and closed arm entries; and total entries into all arms.
- *Social Interaction test.* This test will be conducted in the open-field test arena (as detailed above). A rat that is not participating in the study will be positioned in a small metal grid compartment (10cmX20cmX15cm) in the center of the arena. Following 5 min habituation period for the testing room, rats will be placed at a corner of the arena for 5 min of free exploration. The number of line crossings and the time spent in the central and the peripheral areas will be manually recorded. The total line crossings will represent the activity level of the rat. The ratio between line crossings in the peripheral area and the total line crossings, and the relative time spent, will be considered a measure of anxiety. Additionally, the relative time spent in exploring the animal will

also be recorded. Exploring an animal will be defined by sniffing, approaching the compartment (minimal distance of 1cm) and physical touch with the compartment.

- *Social recognition test.* This test, partially adapted from Kogan, Frankland, and Silva (2000), will be conducted 30 min after the social interaction test, in the same apparatus - the open-field test arena (as detailed above). Two small metal grid compartments (10cmX20cmX15cm) will be positioned in the center of the arena. The rat that was used in the social interaction test will be positioned in one compartment and will serve as the 'familiar' rat, while another novel rat will be positioned in the other compartment and will serve as the 'unfamiliar' rat. Rats will be placed at a corner of the arena for 5 min of free exploration. Activity level and measures of anxiety will be taken as detailed in the social interaction test. Additionally, the relative time spent in exploring each of the positioned animals will also be recorded. Exploring an animal will be defined likewise by sniffing, approaching the compartments (minimal distance of 1cm) and physical touch with the compartments. Comparison between the time spent exploring the 'familiar' and 'unfamiliar' rats will serve as a measure of social recognition.

Behavioral battery B. This behavioral assessment battery will be conducted 4 weeks following the exposure to the SR and UWT and will consist of the octagon test, the elevated zero-maze and another social interaction test and social recognition test:

- *The octagon test.* Consists of a wooden octagon-shaped box 90.0cm × 90.0cm × 38.0cm, positioned in a dimly-lit room. The walls are painted black; the floor is white and divided by 0.5 cm wide black lines into 25 squares 17.0cm × 17.0cm. Following 5 min habituation period for the testing room, rats will be placed at a corner of the box for 5 min of free exploration. The number of line crossings and the time spent in the central and the peripheral areas will be manually recorded. The total line crossings will represent the activity level of the rat. The ratio between line crossings in the peripheral area and the total line crossings, and the relative time spent, will be considered a measure of anxiety.

- *The elevated zero maze.* This maze consists of a circular-shaped platform with two open quadrants and two closed quadrants — surrounded by 38-cm high opaque walls on two sides, with arms of the same type located opposite each other (partially adapted from Singh, Bishnoi, & Kulkarni, 2007). Each rat will be placed on the open quadrant facing a closed quadrant and will be allowed to explore the maze for 5 min, following 5 min habituation period to the testing room. Arm entry will be defined as entering an arm with all four paws. The following measures will be used: durations in open and closed quadrants, open and closed quadrants entries; and total entries into all quadrants.
- *Social interaction test.* This test will be conducted in the same manner as was detailed above in Behavioral battery A, except for the use of a different rat in the compartment.

Social recognition test. This test will also be conducted in the same manner as was detailed above in Behavioral battery A, except for the use of different rats in the compartments.

Biochemical assessments. At the end of the behavioral experiments tissue will be collected and gene expression levels analysis will be examined in brain areas associated with positive or negative affect, including the amygdala, dorsal and ventral hippocampus, medial prefrontal cortex, Nu. Accumbans. We will examine alterations in the expression of CRF receptors, endorphin receptors (Kappa, Mu), GABA receptors (GABAAα1,2,4,6, γ2, GABAB2), GAD65/67, benzodiazepine inhibitory peptide (ACBP), 5HTT(SERT) using commercially available antibodies and quantitative western blotting (Jacobson-Pick et al., 2008). RT PCR will be used to examine the mRNA expression level of CRF receptors, GAD65/67, and peripheral benzodiazepine receptor.

Data Analysis and Statistics

The circadian data regarding body temperature and activity will be first analyzed using Dataquest A.R.T. software (DSI, St. Paul, USA). To test for the effects of SR alone, or with the effect of adulthood stress, on circadian activity and body temperature rhythms as well as on behavioral and

biochemical indices, data will be subjected to one or factorial MANOVA with repeated measures. Additional post-hoc tests will be used as needed. All statistical analyses will be conducted using SPSS 15.0.

Task 5: **The impact of pre-exposure to juvenile stress on the outcome of exposure to UWT** –

Introduction

The impact of juvenile stress on the ability to cope with stress and trauma in adulthood has been demonstrated using different adulthood stressors (Avital and Richter-Levin, 2005; Tsoory and Richter-Levin, 2006; Tsoory et al, 2008). However, its impact on coping with UWT was not yet examined and characterized. As a preparation for the next steps (tasks 6, 7) we wanted to characterize the effects of pre-exposure to juvenile stress on coping with UWT in adulthood.

The UWT has been developed as a unique model of acute robust trauma and has been demonstrated to have long-lasting behavioral consequences with strong face validity to PTSD symptoms (Richter-Levin, 1998; Cohen et al. 2009). More recently an additional dimension has been added to this model – the impact of exposure to a reminder cue, which was found to have clear consequences at the behavioral and electrophysiological levels (Ardi and Richter-Levin, in preparation, and above (task 3)). We continue to investigate the behavioral, electrophysiological and biochemical associated alterations, also in order to establish the baseline for assessment of the effects of predisposing factors later on.

In task 3 the reminder cue was exposing the animal again to swim. Although exposing it to swim is not re-exposure to the UWT, the reminder cue was in a way returning to the trauma scene, not just being exposed to a reminder of it. We thought it would be important to examine whether exposure to a reminder cue, out of the immediate context of the trauma, would have similar effects. Furthermore, in task 3 the effects were examined 24 hrs after the exposure to the UWT. Here we wanted to examine the long term effects of the exposure (4 weeks).

Importantly, in task 5 we have also started to examine the impact of a distal reisk factor – the juvenile stress – on the ability of animals to cope with the UWT in adulthood.

Aims:

The aim of the current study was to test the behavioral effects of the exposure to an odor reminder of UWT 4 weeks following the UWT with or without a previous exposure to juvenile stress.

Methods

Animals

Male Sprague Dawley rats (~22 days old, 30-50 g) were used for the experiments. Animals were housed in groups of ~4, at 22 ± 2°C under 12-h light/dark cycles. Water and food were available ad libitum. The experiments were approved by the University of Haifa Ethics and Animal Care Committees.

Experimental groups

Following acclimation all rats were randomly assigned to one of the following experimental conditions:

1. **Juvenile and UWT stress exposures + odor reminder [J+U(+)]** – Rats were exposed to 'juvenile stress' (ages 27-29 PND's), and in adulthood (at age of 60 days), to 'UWT stress'. 4 weeks folowing the UWT rats were exposed to the odor 'reminder'.
2. **UWT stress + odor reminder [UWT(+)]** – Rats were not exposed to 'juvenile stress', but in adulthood were exposed to 'UWT stress'. 4 weeks following the UWT rats were exposed to the odor 'reminder'.
3. **Juvenile and UWT stress exposures [J+U(-)]** – Rats were exposed to 'juvenile stress' (ages 27-29 PND's), and in adulthood (at age of 60 days), to 'UWT stress'. Rats were not exposed to the odor 'reminder'.
4. **UWT stress [UWT(-)]** – Rats were not exposed to 'juvenile stress', but in adulthood were exposed to 'UWT stress'. Rats were not exposed to the odor 'reminder'
5. **Control [Control]** – Rats were not exposed to 'juvenile stress', and in adulthood were exposed to the odor only without the exposure to 'UWT stress'. 4 weeks following the odor exposure, rats were exposed to the odor 'reminder'.

Experimental design

As depicted in table 1: following delivery and an additional acclimation period of five consecutive days, rats were randomly assigned to the different experimental groups. Rats were either exposed to 'Juvenile- stress' (27-29 PNDs) or not. In adulthood (~60 PND), J+UWT (+) , UWT (+), J+UWT, UWT (-) and Control rats were exposed to 3 consecutive days of habituation to the cage (2 min. per day). On the 4^{th} day, rats' were exposed to an odor and then immediately to the UWT stress. Control rats were exposed to the 3 days of habituation and in the 4^{th} day, rats were exposed to the odor only without the exposure to the UWT stress.

4 weeks following the UWT exposure, J+UWT (+) , UWT (+) and Control rats were re exposed to the odor and then were tested in the Open Field test, 24 hours after the OF test, J+UWT (+) , UWT (+) and Control rats were again exposed to the odor and then were tested in the Elevated Plus Maze. J+UWT and UWT (-) rats were tested in the OF and to the EPM without the exposures to the odor prior to the tests.

Procedures and Ages				
Groups	**Juvenile Stress** [a]	**UWT (+ odor)** [b]	**Odor Re exposure** [c]	**Behavioral Assessments** [d]
PNDs'	*27-29*	*65±*	*90-91±*	
Control (n=24)	-	-	+	+
UWT(-) (n=38)	-	+	-	
UWT(+) (n=42)	-	+	+	
JS+UWT(-) (n=29)	+	+	-	
JS+UWT(+) (n=30)	+	+	+	

Table 1. Experimental design

a. 'Juvenile stress': 3 consecutive days of exposure to acute stressors: 27PND – forced swim stress (10min.), 28PND – elevated platform (30min. X 3, ITI60min. in home cage), and 29PND – confinement (120min.).
b. 'Under water trauma': rats are given 30 s to swim in a water tank (50x60x60 cm) and then are held under water for 30 s, using a special metal net.
c. Odor reminder: After 2 min. habituation to the room, rats were exposed to a vanilla odor inside the cage for 30 sec**.**
d. Behavioral assessments: (1) Open field test: 8 min. testing under dimly light; (2) Elevated plus maze test: 8 min. testing under full light.

Behavioral procedures

'Juvenile stress' protocol

This protocol (Tsoory et al., 2007a; Tsoory et al, 2007b) is an in tandem three-day exposure to different stressors (detailed below) applied during juvenility (ages 27-29 days) at approximately midday (10:00-13:00) - each day a different room.

- Day 1. (27d) Forced swim: 10 min. forced swim in an opaque circular water tank (diameter 0.5m; height 0.5m; water depth 0.4m), water temperature 22±2°C .
- Day 2. (28d) Elevated platform: three 30 min. trials; ITI (Inter-Trials Interval): 60 min in the home cage. Elevated platform: (12X12cm) 70cm above floor level, located in the middle of a room.
- Day 3. (29d) Restraint: rats were placed in a metal mesh restraining box (11X5X4 cm.) that prevented forward-backward movement and limited side-to-side mobility, but did not discomfort

the animal in any other way. Rats remained in the restraining box for 2 hrs under full light illumination.

'Odor Reminder'

After 2 min. habituation to the room, rats were exposed to a vanilla odor inside the cage for 30 sec. The exposure to the odor reminder was conducted in the same way both before the UWT and before the behavioral tests.

'Underwater trauma' stress protocol

The underwater trauma stress was carried out by placing a rat in a plastic tank. Rats were given 5 sec. of free swimming and then were held under water for additional 45 sec, using a special metal net (20X20X15cm.), (adapted from Wang et al., 2000).

All the underwater trauma stress sessions were carried out between 9:00 – 15:00.

Behavioral assessments

Open Field test

The open field test was carried out according to methods described previously (Avital and Richter-Levin, 2005). Briefly, the open field test consist of a square Plexiglas box (50×50×38 cm) positioned in a dimly red-lit ventilated sound-attenuated cupboard. The walls are painted black, the floor is white and divided by 0.3cm-wide black lines into 25 equal squares of 10×10 cm each. At the time of testing, after 5 min. habituation to the room Rats were placed at the corner of the open field facing the wall and were allowed to explore the novel environment for 8 min while their behavior was recorded and analyzed by EthoVision XT8 tracking system.

All rats were tested between 8:00 and 15:00 hours.

Elevated Plus Maze test

The elevated plus maze test was carried out according to methods described previously (Pellow et al., 1985). Briefly, the maze is placed 50 cm above the floor and consists of two open arms and two closed arms (with 30cm high Plexiglas walls and no roof), arranged in a way that similar arms are opposite to each other. At the time of testing, after 5 min. habituation to the room, each animal was placed in the center of the maze facing an open arm and was allowed to explore the arena for an 8 min session. Behavior was recorded and analyzed by EthoVision XT8 tracking system.

All rats were tested between 8:00 and 15:00 hours.

Statistical Analysis

Differences were determined using one-way or repeated measures analysis of variance (ANOVA). All post hoc comparisons were made using Bonferroni multiple comparison test.

Results

Open field test: as depicted in figures 1-ABCD, One way ANOVA indicated a significant main effect for the exposure on number of entries, time spent and distance covered in the center of the arena [$F_{(4,158)}$=2.992, p<0.05.; $F_{(4,158)}$= 2.113, p<0.08; $F_{(4,158)}$= 2.838, p<0.05.], respectively. In addition, One way ANOVA indicated a significant main effect for the exposure on total distance covered in the open field arena [$F_{(4,158)}$= 2.958, p<0.05.]. Further Post hoc comparisons indicated that J+U(+) rats' entered less frequently and covered less distance in the center of the OF arena compared to Control and UWT(+) rat's,(p<0.05/ p<0.08). In addition Post hoc comparisons indicated that J+U(+) spent less time in the center of the open field arena and in total, covered less distance in the OF arena compared to UWT(+) rats', (p<0.05).

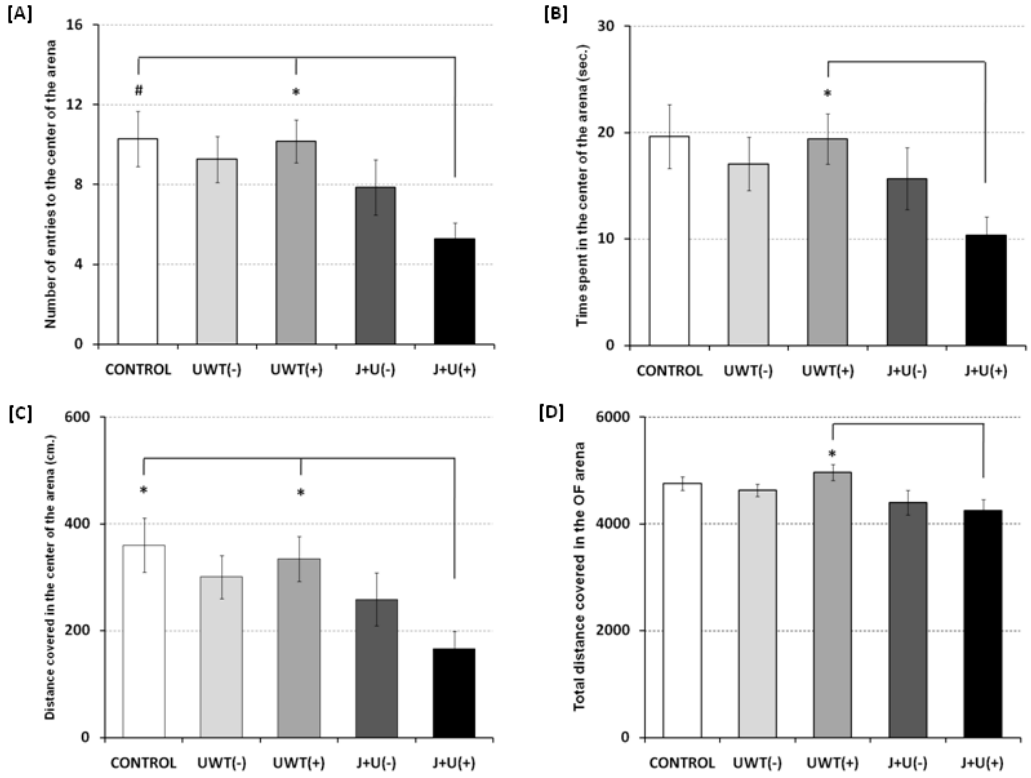

Figure 1-ABCD: Number of entries (A), Time spent (B), Distance covered in the center of the Open Field arena (C) and Total distance covered in the Open field arena (D)**:** An exposure to juvenile stress and to the UWT reminder reduced the number of entries and the distance the rats covered in the center of the OF arena compared to Control and UWT(+) rats'. Additionally, the exposure to juvenile stress and to the UWT reminder also reduced the time spent and the total distance covered in the OF arena compared to UWT(+) rats'. [N: 'Control'- 24, 'UWT(-)'- 38, 'UWT(+)'- 42, 'J+U(-)'- 29, 'J+U(+)' -30].

[***** = p<.05, **#** = p<.08].

Elevated plus maze test: as depicted in figures 2-ABCD, One way ANOVA indicated a significant main effect for the exposure on number of entries, time spent and distance covered in the

open arms of the elevated plus maze [$F_{(4,158)}$=8.925, p<0.001.; $F_{(4,158)}$= 10.421, p<0.001; $F_{(4,158)}$= 8.019, p<0.001.], respectively. In addition, One way ANOVA indicated a significant main effect for the exposure on total distance covered in the EPM arena [$F_{(4,158)}$= 10.586, p<0.001.]. Further Post hoc comparisons indicated that both J+UWT(-) and J+UWT(+) rats' entered less frequently to the open arms of the elevated plus maze and covered less distance in the EPM arena compared to all other groups,(p<0.05/ p<0.08). In addition, while J+UWT(+) rats' showed a reduction in time spent and distance covered in the open arms of the EPM compared to Control, UWT(-) and UWT(+), (p<0.05), J+UWT(-) rats' showed a reduction in these measurements, only compared to Control and UWT(+) rat's, (p<0.05/ p<0.08). Post hoc comparisons also indicated that UWT(-) rats' spent less time in the open arms of the EPM, (p<0.05) and covered less distance in the EPM arena, (p<0.08), compared to Control rats'.

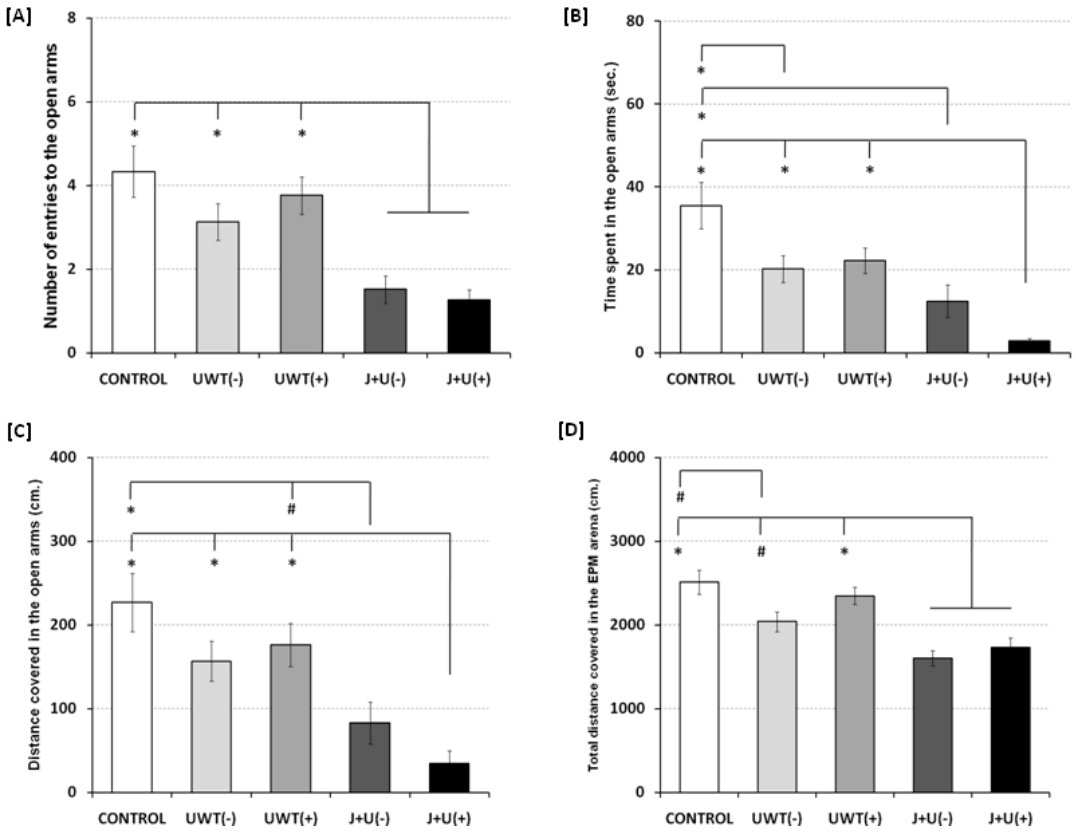

Figure 2-ABCD: Number of entries (A), Time spent (B), Distance covered in the open arms of the elevated plus maze (C) and Total distance covered in the elevated plus maze (D): An exposure to juvenile stress and UWT, with or without an exposure to the UWT reminder reduced number of entries to the open arms and the total distance covered in the EPM arena compared to Control, UWT(-) and UWT(+) rats'. Additionally, while the exposure to juvenile stress and to the UWT reminder reduced both time spent and distance covered in the open arms of the EPM compared to Control, UWT(-) and UWT(+) rats', the exposure to juvenile stress without the UWT reminder reduced time spent in the open

arms, only compared to Control and the distance covered in the open arms compared to Control and UWT(+) rats' only. The exposure to the UWT with the reminder reduced both time spent in the open arms and total distance covered compared to Control Rats'. [N: 'Control'- 24, 'UWT(-)'- 38, 'UWT(+)'- 42, 'J+U(-)'- 29, 'J+U(+)' -30]. [***** = p<.05, **#** = p<.08].

Conclusions

The results confirmed our hypotheses:

- First, by itself, UWT had effects even 4 weeks after the exposure to the trauma, as was indicated by moderate symptoms exhibited by the animals in the Elevated Plus Maze. The longevity if the effects of the trauma are very important for this to be accepted as a PTSD-relevant model.
- Introducing an odor reminder cue, which was not part of the trauma context, during the behavioral tests 4 weeks after the exposure to the trauma, had no added impact by itself.
- Prior exposure of the animals to a distal risk factor (the juvenile stress) had a moderate added impact, as was indicated by more severe symptoms in the Elevated Plus Maze test and a similar tendency in the Open Field test.
- However, prior exposure of the animals to a distal risk factor (the juvenile stress) had a moderate added impact on the effect of the reminder cue. Animals that were exposed to the combination of the distal risk factor and UWT, exhibited, 4 weeks after the exposure to the UWT trauma, significantly more severe symptoms in the presence of the reminder cue, as was indicated by more severe symptoms in both the Elevated Plus Maze test and the Open Field test.
- This experiment was conducted in rounds, with a representation of all the groups in each round. It is important to note that there was high consistency of the results over rounds, which increases the confidence in the results.
- Thus, even before adding the question of the impact of the combination of distal (juvenile stress) and proximal (sleep restriction) risk factors on coping with the trauma, this protocol - of prior exposure to Juvenile test, exposure in adulthood to the UWT and testing even 4 weeks after the trauma in the presence of a reminder cue – is an effective protocol for PTSD-related drug testing, and for neurobiological examination of the neural basis of PTSD.

Task 6: **Assessing juvenile stress pre-disposing effects on sensitivity to sleep restriction in adulthood** –

We will begin to investigate our hypothesis that pre-exposure to a distal risk factor (juvenile stress) increases the vulnerability to the aversive impact of a proximal risk factor (sleep restriction) on coping with stress and trauma in adulthood. Animals exposed to sleep restriction with or without pre-exposure to juvenile stress will be compared.

Experimental Procedure:

Experimental procedures will be similar to those of Task 3 above, with the addition of exposure to a distal risk factor (juvenile stress) (Figure 1).

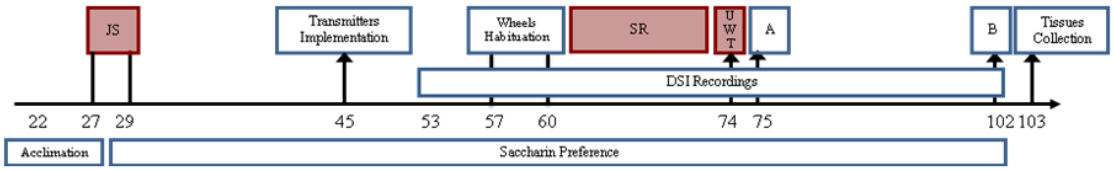

Figure 1 - Investigating the Impact of JS on the SR effect on the response to UWT in adulthood: Rats will arrive at 22 PND. After acclimation they will be evaluated for saccharin preference throughout the experiment (27-102 PNDs). Also at 27 PND, rats will be exposed to the juvenile-stress protocol (27-29 PNDs). At 45 PND they will be implanted with DSI transmitters. Activity and body temperature will be recorded starting after recovery of 7 days and until 102 PND. After 3 days of wheels habituation (1 hr every day), at 60 PND rats will be exposed to sleep restriction (SR) for 15 days at the longest. Immediately following the SR rats will be exposed to the underwater trauma (UWT) and two hours following the UWT, blood samples will be taken from the tails. An assessment of the animals' behavioral performances will be conducted at 75 PND and at 102 PND, using behavioral batteries A and B respectively. At 103 PND, all animals will be decapitated and blood and relevant brain areas will be harvested.

Tasks 3 and 6 will be run in parallel in order to reduce the number of animals required for some of the control groups.

Key research accomplishments

While this is only the initial stage of the project, the following can already be indicated as research accomplishments:

- The UWT model, which is an ethological model of a brief but intense traumatic event (Richter-Levin, 1998) was further developed here in a way that is of particular relevance to combat soldiers. It was found to have an impact by itself, but to be a convenient platform for examining the added impact of relevant risk factors.
- The maladaptive response of PTSD patients to reminder cues of the traumatic events is a hallmark of the disorder. We have established an effective animal model that is sensitive to the effects of the reminder cue. This will enable us
  a) To utilize the sensitivity of the model as a drug testing platform.
  b) To better understand variables which contribute to the effectiveness of reminder cues (in order to guide treatment).
  c) To use the model to elucidate the neural mechanisms associated with abnormal responses to reminder cues.
- A rat model of high relevance to PTSD was confirmed (task 5). That finding that PTSD symptoms in this model last for over four weeks establishes it as a relevant model but also enables utilizing this model for long-term drug treatment at different time points following the exposure to the traumatic event.

Reportable outcomes

Manuscripts in preparation:

1) Ardi Z. and Richter-Levin G., Re-exposure to a trauma reminder affects local circuit activity and LTP induction in the rat dentate gyrus.
2) Horovitz, O., Tsoory, M.M, Yovell, Y., and Richter-Levin, G., A rat model of pre-puberty (Juvenile) stress-induced predisposition to stress-related disorders: Sex similarities and sex differences in effects and symptoms.
3) Ritov, G. Ardi, Z., and Richter-Levin, G., Differential activation of dorsal and ventral hippocampus and amygdala following an exposure to a reminder of underwater trauma.

Abstracts in meetings:

1) Ardi Z. and Richter-Levin G., Synaptic and local circuit plasticity in the dentate gyrus. Potential relevance to traumatic memories. In: the 8th IBRO world congress of neuroscience. Italy, 14-18. July 2011.
2) Ardi Z., Richter-Levin A., and Richter-Levin G., 'Juvenile stress' exacerbates the impact of an exposure to an odor reminder of a traumatic experience in adulthood. In: The 16th Annual meeting of the Israeli society for biological psychiatry. Israel, 20-22 March, 2012.
3) Horovitz, O., Strominger, I., Ashkenazi-Karni, S., and Richter-Levin, G., Exposure to stress differentially affects behavior and brain activity in male and female rats. In: The 20th Annual meeting of the Israel society for neuroscience, Israel, December 2011.
4) Horovitz, O., Strominger, I., Ashkenazi-Karni, S., and Richter-Levin, G., Exposure to stress differentially affects behavior and brain activity in male and female rats. In: The 16th Annual meeting of the Israeli society for biological psychiatry, Israel, 2012.

Conclusions

This report is of the first year of a 4 years project. During this year we have

a) Established infrastructure that is critical for the execution of the project.
b) Have trained postdocs and students with the methodologies required for the conductance of the project.
c) Completed the initial phase of the experiments that establish the basis for the second year's part.
d) Have already obtained important scientific findings that are of relevance to understanding PTSD.

Thus, while it is too early for conclusions we can summarize the first year as a fruitful year and can look forward to further achievements in the coming years.

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